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Chemical defenses of the tropical, benthic marine cyanobacterium *Hormothamnion enteromorphoides*: Diverse consumers and synergisms

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Abstract

The tropical, benthic marine cyanobacterium *Hormothamnion enteromorphoides* periodically dominates shallow reef habitats, forming erect tufts similar in size to the thalli of macroalgae. Although periodically very common and lacking structural defenses, *H. enteromorphoides* seems to be a low preference food item for marine herbivores. An organic extract of *H. enteromorphoides* deterred feeding by a natural assemblage of reef fishes, suggesting that *H. enteromorphoides* was chemically defended. Bioassay-guided fractionation of the organic extract led to a mixture of related cyclic peptides, with the known compound laxaphycin A as the major component. The peptide mixture significantly and strongly deterred feeding at natural concentrations by the parrotfish *Scarus schlegeli*, the sea urchin *Diadema savignyi*, and the crabs *Leptodius* spp., but had a less striking and nonsignificant effect on feeding by the pufferfish *Canthigaster solandri*. Previous studies found that laxaphycins exhibited synergistic antifungal and cytotoxic effects. However, when tested at equal concentrations in feeding assays with *S. schlegeli* and *C. solandri*, laxaphycin A was as effective at deterring feeding as was the peptide mixture, indicating that the peptides do not interact synergistically to deter herbivory.

Benthic cyanobacteria (blue-green algae) are a ubiquitous component of coral reef communities. Often cyanobacteria are present as part of a mixed-species “algal turf” assemblage; however, a number of genera (e.g. *Hormothamnion*, *Microcoleus*, *Schizothrix*) periodically undergo “blooms” when they become common and may even dominate large patches of shallow reef habitat (pers. obs.). During blooms, cyanobacteria may form erect tufts similar in size to the thalli of macroalgae. Dense blooms of cyanobacteria potentially could provide a rich source of food for marine herbivores; however, little is known about cyanobacteria–herbivore interactions in the marine benthos.

Cyanobacteria have been a productive source of novel secondary metabolites (Moore 1981; Faulkner 1995, and references therein). The secondary metabolites of marine benthic cyanobacteria represent a diversity of structural classes and, in striking contrast to the secondary metabolites of marine macroalgae, often contain nitrogen (Moore 1981). Some secondary metabolites from marine cyanobacteria have been examined for pharmacologic activity (e.g. Fujiki et al. 1981, 1982; Eliasson et al. 1983; Suganuma et al. 1984; Fujiki and Sugimura 1987; Gerwick et al. 1994), but little attention has been paid to their roles in nature. The unpalatability of some

planktonic, freshwater cyanobacteria has been linked to their secondary chemistry (e.g. Moore 1981; Fulton and Paerl 1987; Carmichael et al. 1990; DeMott et al. 1991; Carmichael 1992; Gilbert 1996), and the effect on potential consumers of secondary metabolites from the tropical, benthic marine cyanobacterium *Microcoleus lyngbyaceus* (= *Lyngbya majuscula*) has been examined in detail (Wylie and Paul 1988; Paul et al. 1990, 1993; Paul and Pennings 1991; Pennings and Paul 1993; Pennings and Carefoot 1995; Pennings et al. 1996). Otherwise, little is known about how cyanobacterial secondary metabolites mediate ecological interactions. The most obvious potential role for secondary metabolites in benthic organisms like seaweeds, and the one that has received the most attention in a variety of benthic taxa, is reducing damage by consumers (Paul 1992); however, additional roles probably exist (Paul and Fenical 1986; Schmitt et al. 1995).

This study focuses on the tropical marine cyanobacterium *Hormothamnion enteromorphoides*, from the family Nostocaceae. On Guam, we periodically observe dense blooms of *H. enteromorphoides* that dominate hundreds of meters of reef flats (pers. obs.). Despite being very abundant at times, *H. enteromorphoides* seems to attract little attention from common fish and invertebrate herbivores (pers. obs.). A collection of *H. enteromorphoides* from Puerto Rico contained a series of at least 15 related cyclic peptides termed hormothamnins (Gerwick et al. 1989, 1992). The hormothamnins are very closely related to a similar series of cyclic peptides, the laxaphycins, isolated from the terrestrial cyanobacterium *Anabaena laxa*, also from the Nostocaceae (Frankmölle et al. 1992a,b). The laxaphycins exhibited synergistic effects when tested in antifungal or cytotoxicity assays: a mixture of all the laxaphycins, or of laxaphycin A and B, was more active than any single laxaphycin tested at the same total concentration (Frankmölle et al. 1992b).

We were interested in *H. enteromorphoides* for several reasons. We suspected that this cyanobacterium might be chemically defended because it appeared to be a low-pref-

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erence food for herbivorous fishes and gastropods even though it was neither tough nor calcified (Pennings and Paul 1992; pers. obs.). In a survey of 11 species of cyanobacteria, seaweeds, and seagrasses, an organic extract of *H. enteromorphoides* was one of the few extracts that deterred feeding by the sea hare *Dolabella auricularia* (Pennings and Paul 1992), again suggesting that a potent chemical defense was present. Based on the studies by Gerwick et al. and Frankmölle et al., we speculated that Pacific Ocean populations of *H. enteromorphoides* might contain cyclic peptides and that these peptides might act synergistically to deter consumers. We asked three general questions. (1) Was *H. enteromorphoides* from Guam chemically defended against a variety of potential consumers? (2) If so, were cyclic peptides similar to the hormothamnins or laxaphycins responsible? (3) If so, did these peptides interact synergistically to deter consumers?

Methods

Cyanobacterium secondary chemistry—We collected *H. enteromorphoides* from reef flats in the vicinity of Ypan, Guam (13°22'N, 144°47'E), in January and July 1993. *H. enteromorphoides* forms bright green, soft, mucilaginous tufts that were easily collected by hand. *H. enteromorphoides* was repeatedly extracted in 1:1 dichloromethane:methanol and the organic layer reduced to dryness in a rotary evaporator. These bulk extractions were not exhaustive, contained sand and other inorganic material, and produced organic extract yields of 1.1 and 2.1% of dry mass. More exhaustive extractions of three other small collections produced organic extract yields of 1.8, 2.1, and 3.9% of dry mass. We based the concentrations of fractions tested in many of our feeding experiments on a 3% total yield to represent the higher end of the natural range.

One experiment used an aqueous methanol (MeOH) fraction obtained by partitioning the organic extract between 90% aqueous MeOH and hexanes. This procedure removed nonpolar materials such as triglycerides and sterols into the hexane fraction ($\leq 20\%$ of the total extract), leaving a partially purified MeOH fraction containing more polar material.

Most of our feeding experiments used fractions obtained through a series of fractionation steps starting with vacuum flash chromatography, followed by column chromatography and then high-performance liquid chromatography. Rather than look directly for cyclic peptides, we used a bioassay-guided fractionation procedure to isolate the metabolites that made *H. enteromorphoides* unpalatable.

Crude organic extract was fractionated with vacuum silica gel flash chromatography by using six solvent mixtures of increasing polarity [100% hexanes, 10% ethyl acetate (EtOAc)/hexanes, 25% EtOAc/hexanes, 50% EtOAc/hexanes, 100% EtOAc, 100% MeOH]. Based on the results of feeding assays described below, the three most polar fractions were combined and fractionated further, guided by feeding assays, using normal and then reverse-phase (C_{18}) stationary phases (prepackaged cartridge columns [Mega Bond Elut] with gravity flow). The final active fraction was

a polar, off-white crystalline material. A 500-MHz proton-NMR analysis indicated that this material was a semipurified mixture of several cyclic peptides similar to the laxaphycins and hormothamnins.

The cyclic peptides were further purified by reverse-phase HPLC (C_{18} stationary phase, 20% H_2O /MeOH mobile phase) to yield pure laxaphycin A ($\sim 50\%$ of the peptide fraction) as the major metabolite (identified by comparing 500-MHz proton-NMR traces with spectra of standards of laxaphycin A and hormothamnin A) and a mixture of three to four major related cyclic peptides that did not readily separate. We did not find hormothamnin A in our collections of *H. enteromorphoides*.

Potential consumers—We conducted feeding assays with natural assemblages of reef fish in the field and with four consumers in the laboratory. Field assays offered the advantages of simplicity, a natural setting, and a natural, diverse group of fishes and were conducted at Western Shoals in Apra Harbor, Guam (13°25'N, 144°55'E). This shallow (~ 7 -m water depth) reef harbors a diverse group of fishes that fed readily in our assays. In contrast, laboratory assays allowed us to confine tests to specific consumers and to look for between-consumer variation in sensitivity. We conducted laboratory feeding experiments with the parrotfish *Scarus schlegeli*, the sea urchin *Diadema savignyi*, the spotted sharpnose pufferfish *Canthigaster solandri*, and intertidal xanthid crabs *Leptodius* spp. Consumers were either used freshly collected from the field or were fed ad libitum between assays.

S. schlegeli is common on shallow reef flats (Myers 1989). We collected small (8–14-cm) individuals by barrier and dip net from Apra Harbor, Guam. Fish were held and tested in pairs in 20-liter aquaria with running seawater.

D. savignyi is common in high-relief areas of shallow reef flats. We collected *D. savignyi* by hand from the Pago Bay reef flat. Urchins were held and tested individually in 20-liter flowthrough containers submerged in large outdoor aquaria supplied with running seawater.

C. solandri ranges from the reef flat to depths of at least 36 m, feeding on filamentous algae and benthic invertebrates (Myers 1989). We collected *C. solandri* by dip net from Pago Bay, Guam. Fish were held and tested individually in 4-liter flowthrough containers submerged in water tables supplied with running seawater.

Leptodius spp. were collected by hand from the intertidal and shallow subtidal (< 1 m at low tide) zones of Pago Bay. A sample of six representative animals contained only the morphologically similar *L. sanguineus* (H. Milne Edwards, 1834) and *L. nudipes* (Dana, 1852), which are the only species in the genus known from this type of habitat on Guam (R. Kropp pers. comm.). Crabs were held and tested individually outdoors in shallow plastic bowls (~ 30 -cm diam, 6-cm water depth). Water was replaced every 24–48 h.

Feeding assays—The effect of the crude organic extract and the various fractions on feeding was examined in the field. A variety of damselfishes, wrasses, triggerfish, and unicornfish fed during these assays. The extract or fractions were incorporated into an artificial diet (Pennings et al.

1994) consisting of carrageenan (2.5 g), paraffin wax (2.0 g), Kruse catfish food (a commercial fish food for aquaculture, Kruse Grain and Milling, 5.0 g), and fresh water (80 ml). The ingredients were mixed, heated to boiling, and poured into a mold to form small ($\sim 1 \text{ cm}^3$) cubes. Experimental foods contained the extract or fraction that was dissolved in a small amount of ether and added to the recipe after heating but before pouring. Control foods contained identical amounts of ether to control for any effect of ether on feeding; however, the bulk of the ether appeared to quickly evaporate from the food mixture. Pieces of food were attached to polypropylene lines ($n = 4$ pieces per line). Lines with treated and control food were paired and were attached to the reef within 20 cm of each other. Lines were placed and feeding observed using SCUBA. After fish had removed about half of the pieces of food from a pair (i.e. four of eight, this took $\sim 1\text{--}20$ min depending upon the number of fish present and their feeding rates), the paired ropes were retrieved and the number of food pieces that had been completely eaten was scored. Data were analyzed with Wilcoxon's signed rank test. Occasional ties were dropped as is required by the calculations (final sample sizes were 19–20; two-tailed P -values are reported).

The effect of the aqueous MeOH fraction, the peptide mixture, and pure laxaphycin A on feeding was examined in the laboratory with *S. schlegeli* and/or *D. savignyi*, using methods similar to Hay et al. (1994). The artificial diet consisted of 0.72 g agar, 4 g freeze-dried *Enteromorpha* spp. (a highly palatable green alga), and 36 ml water. The recipe was heated and extracts added as above, then poured into a narrow rectangular mold placed over a strip of fiberglass window screen. Treated and control foods were placed in parallel bands on the same piece of screen, which was then cut to produce replicate strips of screen, each with a rectangle of treated and of control food. Herbivores were allowed to feed until approximately half of the mesh squares originally covered by food had been exposed. The number of mesh squares completely exposed was compared between the treated and control foods using a paired t -test. Occasional replicates in which consumers either refused to eat or rapidly ate all the food provided no information on relative palatability of diets and were dropped. Final sample sizes ranged from 8 to 13. Two-tailed P -values are reported.

The effect of the peptide mixture and pure laxaphycin A on feeding was further examined in the laboratory with *C. solandri* and *Leptodius* spp. The artificial diet (Pennings et al. 1994) consisted of carrageenan (2.5 g), Prime Reef mix (a commercial frozen aquarium fish food, Ocean Nutrition, wet mass 35 g), and water (50 ml). The recipe was heated and extracts added as above and poured into a mold to form small ($\sim 1 \text{ cm}^3$) cubes. Individual consumers were each offered one piece of treated and one piece of control food. Paired no-consumer trials were conducted to estimate changes in the mass of food in the absence of consumption. Individual replicates and paired controls were terminated after approximately half the total food was eaten. Consumer and no-consumer trials were paired by the length of time that foods were immersed in water. Food pieces were weighed before the experiment, then blotted dry and

weighed again after the experiment. Occasional replicates in which consumers either refused to eat or rapidly ate all the food provided no information on relative palatability of diets and were dropped. Final sample sizes ranged from 13 to 16. Data were analyzed following Peterson and Renaud (1989): the variable [change in mass of treated food] – [change in mass of control food] was compared for consumer and no-consumer trials with a paired t -test. Two-tailed P -values are reported.

Results

The crude organic extract of *H. enteromorphoides* strongly deterred feeding by reef fishes when tested at Western Shoals at a concentration of 3% of dry mass of the diet (Fig. 1A). The extract had little or no effect on feeding when tested at the lower concentration of 1.1% of dry mass. Similarly, the aqueous MeOH partition of the crude organic extract significantly deterred feeding by *S. schlegeli* in the laboratory at a concentration of 3% of dry mass, but not at 2% of dry mass (Fig. 1B).

Only one of six flash column fractions deterred feeding by reef fishes when tested at Western Shoals at a combined concentration of 3% of dry mass (Fig. 2). The active fraction (100% EtOAc) was near the polar end of the fractionation scheme, providing our first clue that we might be dealing with secondary metabolites similar to the polar cyclic peptides that had previously been reported from *H. enteromorphoides*. The polar fractions were recombined and separated on a normal phase column with EtOAc and MeOH/EtOAc mobile phases into three fractions (100% EtOAc, 10% MeOH/EtOAc, and 30% MeOH/EtOAc); only the 30% MeOH/EtOAc fraction deterred feeding by reef fishes (data not shown; $n = 20$, $P = 0.87$; $n = 20$, $P = 0.41$; $n = 19$, $P = 0.001$, respectively). The 30% EtOAc/MeOH fraction was further purified on a C_{18} reversed-phase column with H_2O /MeOH and MeOH mobile phases into two fractions (20% H_2O /MeOH, 100% MeOH); only the 20% H_2O /MeOH fraction deterred feeding by reef fishes (data not shown; $n = 20$, $P = 0.03$; $n = 19$, $P = 0.20$, respectively). Proton-NMR analysis of the final active fraction, an off-white crystalline substance, revealed that it was a mixture of four to five major cyclic peptides similar to the hormothamnins and laxaphycins. Based on our yields, the peptide mixture occurred in *H. enteromorphoides* at $\sim 0.3\%$ of dry mass.

The peptide mixture deterred feeding by the parrotfish *S. schlegeli* in the laboratory at a concentration of 0.3% but not at 0.2 or 0.15% (Fig. 3A). The peptide mixture strongly deterred feeding by the sea urchin *D. savignyi* at both 0.2 and 0.3% concentrations (Fig. 3B). Pure laxaphycin A deterred feeding by the parrotfish *S. schlegeli* at both 0.2 and 0.3% (Fig. 4). When compared directly, parrotfish did not significantly discriminate between a diet containing 0.2% laxaphycin A and a diet containing 0.2% peptide mixture, although there was a trend toward eating less of the laxaphycin A diet (Fig. 4).

The peptide mixture strongly deterred feeding by the crabs *Leptodius* spp. but did not significantly affect feeding by the

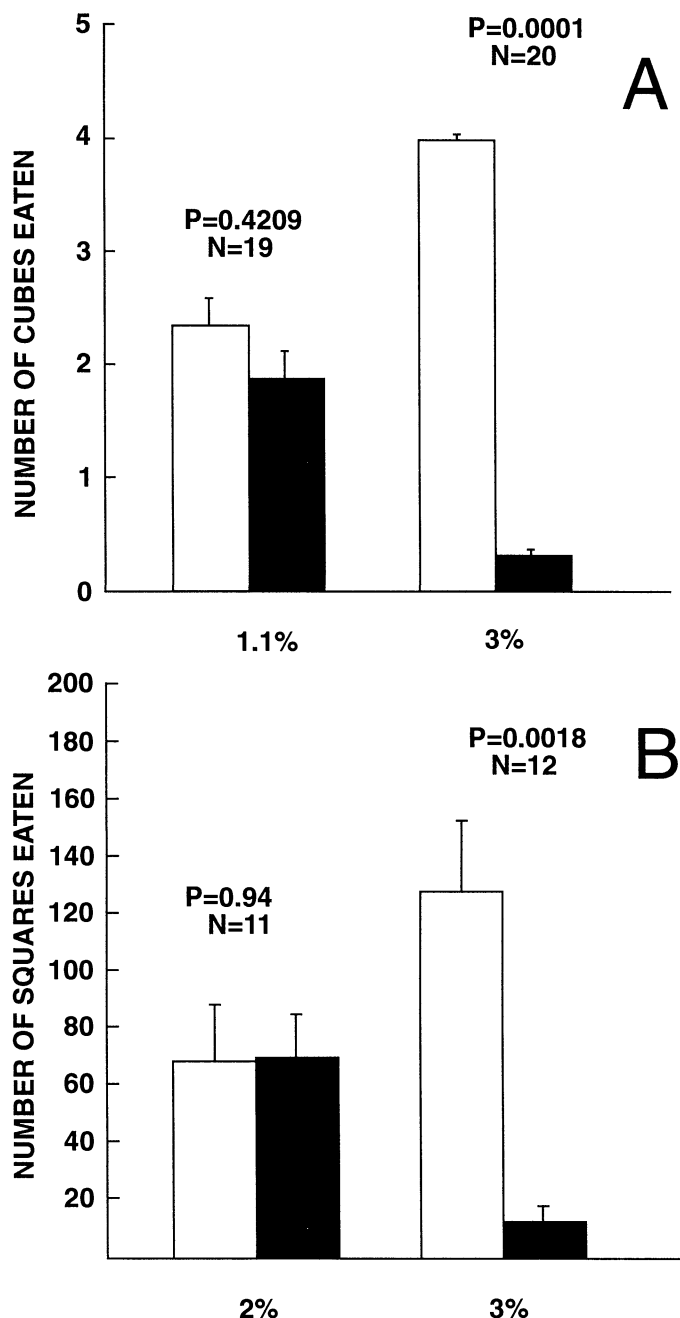


Fig. 1. Effect of two concentrations of (A) crude extract from *Hormothamnion enteromorphoides* on feeding by reef fishes (□, control; ■, crude extract) and (B) aqueous MeOH partition of crude extract from *H. enteromorphoides* on feeding by parrotfish, *Scarus schlegeli*, in the laboratory (□, control; ■, MeOH partition). Data are means \pm 1 SE; sample sizes and *P*-values are given above bars.

pufferfish *C. solandri* at a concentration of 0.3% (Fig. 5). Laxaphycin A showed a marginally significant ($P = 0.057$ with a two-tailed test) trend toward deterring feeding by pufferfish at a concentration of 0.3% (Fig. 5); however, the proportional reduction in feeding was considerably less for pufferfish than for the other three consumers offered laxaphycin A or the peptide mixture.

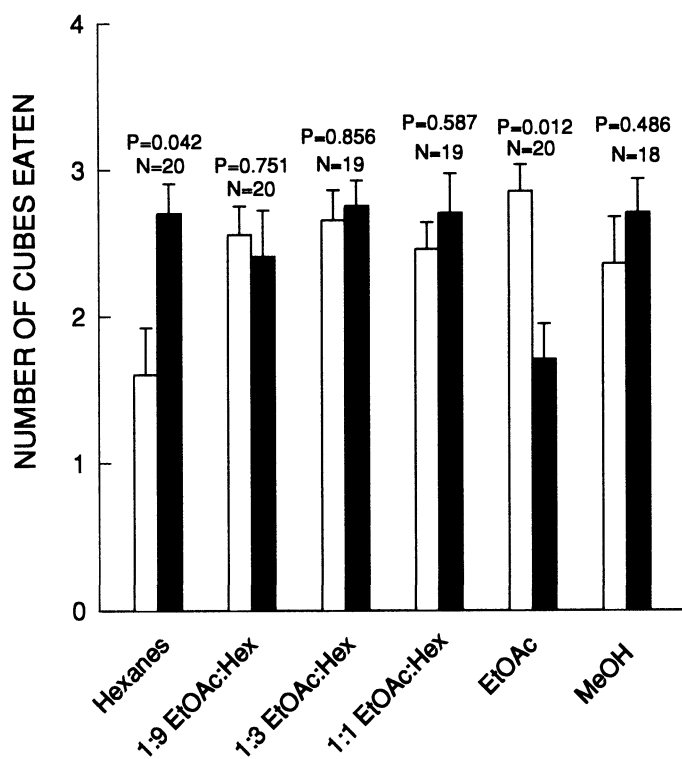


Fig. 2. Effect of six fractions of crude extract from *Hormothamnion enteromorphoides* on feeding by reef fishes. Data are means \pm 1 SE; sample sizes and *P*-values are given above bars (□, control; ■, treated).

Discussion

Our results suggest that *H. enteromorphoides* is chemically defended against most, but not all, potential consumers. Its chemical defenses probably are a key factor allowing *H. enteromorphoides* to bloom and persist in high abundances on the reef despite being neither tough nor calcified. Our bioassay-guided fractionation approach led us directly to a group of related cyclic peptides, with laxaphycin A as a major component. Both the mixture of peptides and pure laxaphycin A were effective at deterring feeding in our assays, and these compounds probably explain most or all of the unpalatable nature of *H. enteromorphoides*, because fractions that did not contain these peptides never deterred feeding.

Our earlier work testing the crude extract of *H. enteromorphoides* against the sea hare *Dolabella auricularia* (Pennings and Paul 1992) used, in retrospect, an unrealistically high crude extract concentration (10% vs. 3% in this study). Nevertheless, *D. auricularia* avoided eating *H. enteromorphoides* despite eating most other soft, uncalcified seaweeds and cyanobacteria (Pennings and Paul 1992), and it is likely that the chemical defenses of *H. enteromorphoides* were the reason why.

Variation between consumer species in sensitivity to chemical defenses is thought to be an important factor governing variation in dietary choices and the evolution of chemical defense strategies (Hay et al. 1988; Hay and Fenical 1992; Hay and Steinberg 1992). The effects of the *H.*

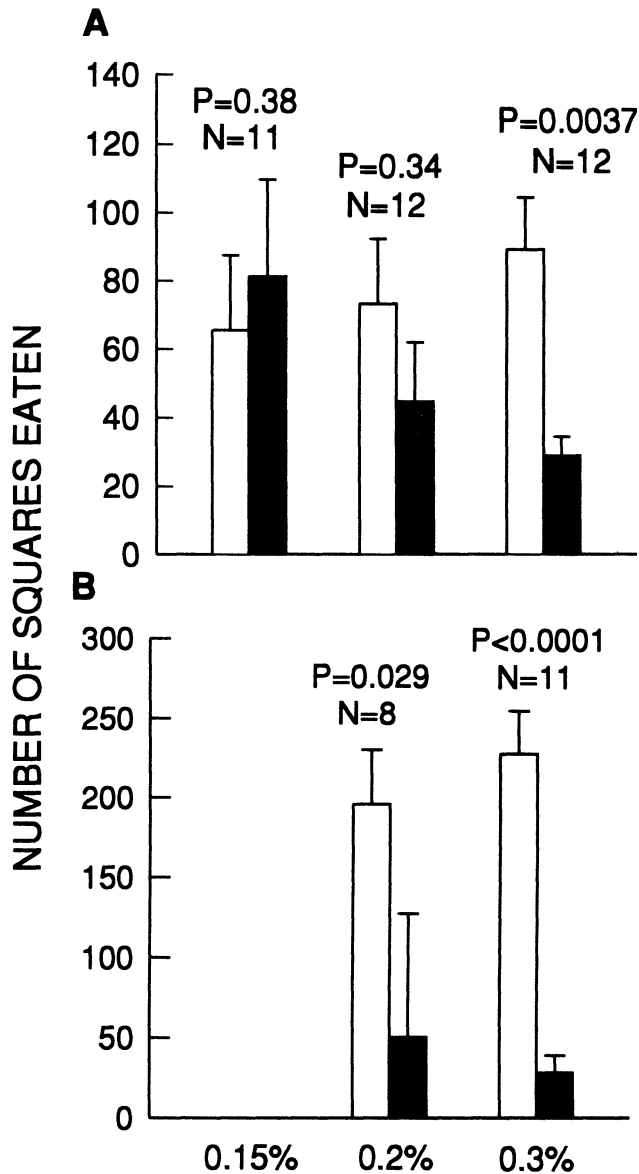


Fig. 3. Effect of three concentrations of peptides from *Horothamnion enteromorphoides* on feeding by (A) parrotfish, *Scarus schlegeli*, and (B) sea urchins, *Diadema savignyi*, in the laboratory. Data are means ± 1 SE; sample sizes and *P*-values are given above bars. □, control; ■, peptides.

enteromorphoides peptide mixture on feeding differed between consumer species in our experiments. First, although pufferfish showed a trend toward being deterred from feeding by the 0.3% peptide mixture, this trend was moderate and not significant. In contrast, all of the three other consumers were strongly and significantly deterred from feeding at this concentration. The greater sensitivity of crabs than pufferfish was surprising because in two previous studies *Leptodius* spp. were consistently less sensitive to secondary metabolites than was *C. solandri* (Pennings et al. 1994; Pennings et al. 1996). Second, sea urchins appeared slightly more sensitive to the peptide mixture than did parrotfish, as sea urchins were deterred from feeding by both 0.2 and 0.3%

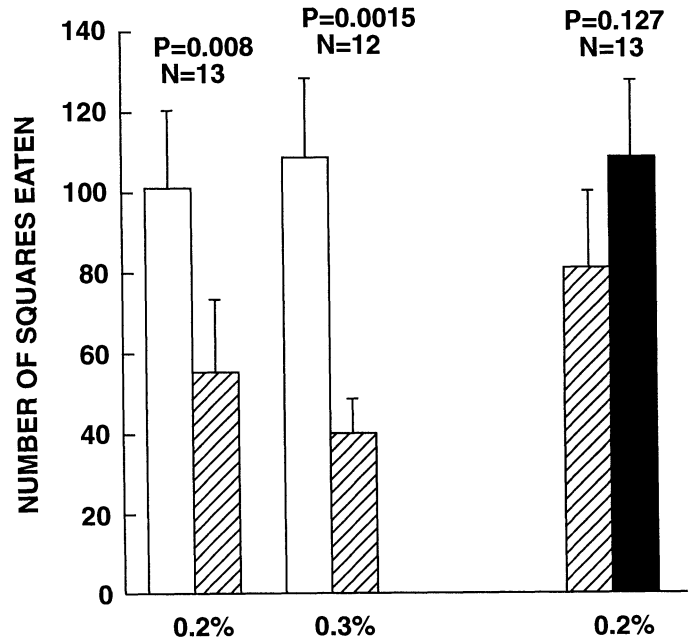


Fig. 4. Effect of two concentrations of pure laxaphycin A on feeding by parrotfish, *Scarus schlegeli*, in the laboratory. Left: laxaphycin A vs. control food. Right: laxaphycin A vs. peptide mixture. Data are means ± 1 SE; sample sizes and *P*-values are given above bars. □, control; ▨, laxaphycin A; ■, peptides.

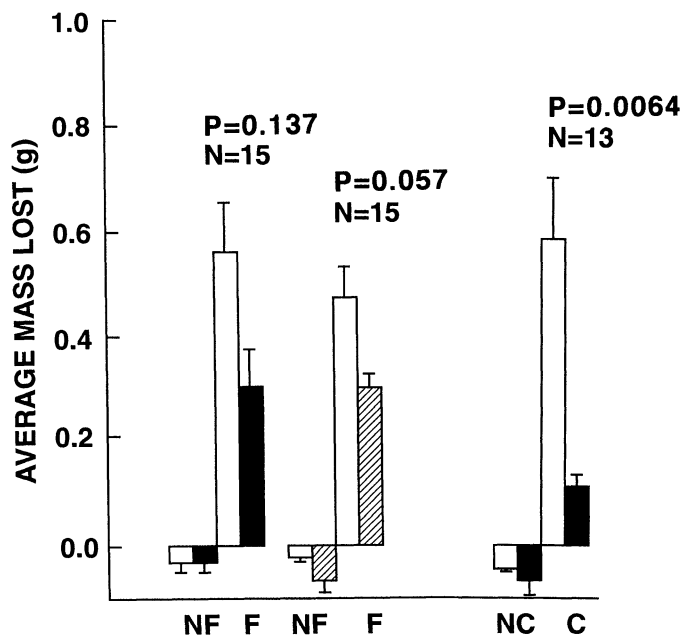


Fig. 5. Effect of peptide mixture and pure laxaphycin A on feeding by crabs, *Leptodius* spp., and pufferfish, *Canthigaster solandri*. F = fish, NF = no fish, C = crab, NC = no crab. Data are means ± 1 SE; sample sizes and *P*-values are given above bars. □, control; ■, peptides; ▨, laxaphycin A.

peptide concentrations but parrotfish only by the higher concentration. These results emphasize the advantages of testing putative chemical defenses against a variety of consumers: had we used only pufferfish, we would have concluded that *H. enteromorphoides* is only weakly or not chemically defended; had we used only sea urchins, we would have concluded that *H. enteromorphoides* has extremely potent chemical defenses. Instead, by using four consumers, we arrive at a more moderate and accurate conclusion: *H. enteromorphoides* is chemically defended against most, but not all, potential consumers. This conclusion is perhaps the most interesting, because it raises the questions of why some consumers are relatively resistant to certain secondary metabolites (from both physiological, ecological, and evolutionary perspectives) and of how herbivore resistance to secondary metabolites affects community interactions and structure.

Both this study and those by Gerwick et al. (1989, 1992) indicated that *H. enteromorphoides* contains numerous closely related peptides. Why not simply produce a single peptide? Natural selection might favor producing a mixture of secondary metabolites if these metabolites interacted synergistically so that the mixture was more active than any single metabolite would be at the same concentration. The laxaphycins display synergistic antifungal and cytotoxic effects (Frankmölle et al. 1992b); however, we found no evidence with our peptide mixture for synergistic effects on palatability. In experiments with both parrotfish and pufferfish, the pure compound laxaphycin A was as effective at deterring feeding as was a mixture of peptides at the same concentration (and perhaps was marginally better). The most likely explanation for these results is that the toxic and unpalatable aspects of the metabolites derive from different physiological pathways. Thus, a synergism affecting toxicity would not necessarily affect palatability. This argument is supported by the observation that the toxic effects of secondary metabolites often correlate poorly with their effects in feeding assays (Hay et al. 1987; Sammarco and Coll 1992; Schulte and Bakus 1992; Pawlik et al. 1995).

If the metabolites do not have synergistic effects on palatability, why does *H. enteromorphoides* produce so many? Why not simply produce more laxaphycin A and nothing else? One possibility is that consumers differ in their sensitivity to particular peptides, so that a mixture will deter a broader range of consumers than would any single peptide. Alternatively, because secondary metabolites probably serve a variety of functions in nature (Paul and Fenical 1986; Schmitt et al. 1995), it may be that the synergistic antifungal and cytotoxic properties of the laxaphycins (Frankmölle et al. 1992a) enhance their effectiveness in other natural roles.

Our laboratory group has now examined in some detail the chemical ecology of two tropical, benthic marine cyanobacteria, *Microcoleus lyngbyaceus* and *H. enteromorphoides*. Both species are periodically common in coral reef habitats around Guam, are unpalatable to generalist herbivores, and contain N-based secondary metabolites that effectively deter feeding by potential generalist consumers (Pennings et al. 1996; this paper). We suggest that unusual N-based secondary metabolites, rare in marine macroalgae, are a major factor allowing these cyanobacteria, which lack other de-

fenses such as toughness or calcification, to occupy reef habitats subject to high levels of herbivory.

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